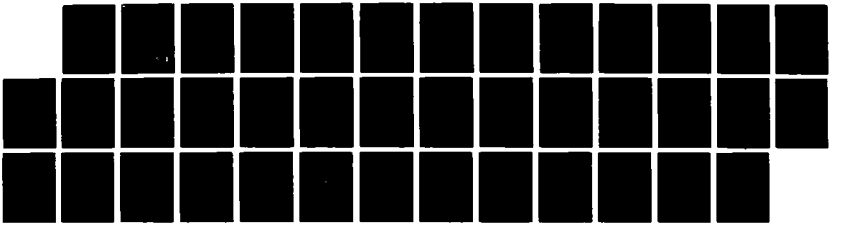
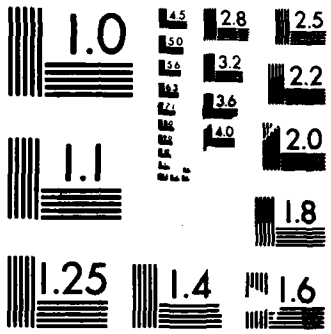
AD-A183 107 TOLERANCE FOLLOWING ORGANOPHOSPHATE POISONING OF TRACHEAL MUSCLE(U) MISSISSIPPI UNIV MEDICAL CENTER JACKSON DEPT OF PHARMACOLOGY AND TOXICOLOGY J M FARLEY ET AL. 08 NOV 85 1/1

UNCLASSIFIED F/G 6/11 NL





MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AD_____

Tolerance Following Organophosphate Poisoning of Tracheal Muscle

Annual Report

Jerry M. Farley
Terry M. Dwyer
November 8, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Fredrick, Maryland 21701-5012

Contract No. DAMD17-83-C-3248

Dept. of Pharmacology and Toxicology
Univ. of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216-4505

SECURITY CLASSIFICATION OF THIS PAGE

AD-A183 107

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): 57640	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Dept. of Pharmacology and Toxicology, Univ. Miss. Med. Ctr. / 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 2500 North State Street, Jackson, Mississippi 39216-4505	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command / 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-83-C-3248
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M1-62734A875; TASK NO. AA; WORK UNIT ACCESSION NO. 445

11. TITLE (Include Security Classification)
TOLERANCE FOLLOWING ORGANOPHOSPHATE POISONING OF TRACHEAL MUSCLE

12. PERSONAL AUTHOR(S)
Farley, Jerry M., Dwyer, Terry M

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 9/15/84 TO 9/14/85	11/8/85	

16. SUPPLEMENTARY NOTATION Abstract presented at FASEB meeting, Anaheim, CA, April 1985. Abstract to be presented at Biophysical Society meeting, San Francisco, CA, February 1986.

17. *CONT'D COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	15	
06	01	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Trachea, swine, muscarinic receptors, tolerance, organophosphate, DFP, contraction, electrophysiology

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Chronic reduction in acetylcholinesterase activity leads to accumulation of acetylcholine at synapses. The continual presence of acetylcholine induces changes in sensitivity of tissues to acetylcholine, in part due to down-regulation (decreased number) of muscarinic receptors. The following report describes experiments designed to determine whether tracheal muscle muscarinic receptors and the tissue response to activation of these receptors change with subacute exposure to diisopropylfluorophosphaonate (DFP). Muscarinic receptor binding characteristics on control and DFP-treated animals, and isometric tension developed in response to various agents (acetylcholine, DFP, bethanechol, K^+) and membrane potential of muscle cells were determined in control and subacutely animals treated with DFP.

Displacement of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding by agonists and antagonists suggests that the tracheal muscle receptors are of the M_2 subtype. Agonists induce a state change in the receptor to a lower affinity form. Subacute treatment with DFP results in a decrease in receptor number of 70% after 7 days and 95% decrease in acetylcholinesterase

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Virginia M. Miller	22b TELEPHONE (Include Area Code): 301/663-7325 / 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete — SECURITY CLASSIFICATION OF THIS PAGE

*17. CONT'D COSATI CODES

FIELD	GROUP
06	16

*19. CONT'D ABSTRACT

activity.

The dose-response relationships for contraction induced by acetylcholine or bethanechol show several shifts in animals treated for up to 14 days with DFP. In vitro treatment with DFP in control tissue causes a spontaneous development of tension which can be blocked by atropine and pirenzepine and marginally by mecamylamine, but is unaffected by tetrodotoxin (TTX).

In vitro DFP treatment shifts the dose-response for acetylcholine to the left in both control and DFP-treated animals. The dose-response curves after in vitro DFP are much steeper in 7 and 14 day DFP-treated animals than in control, 1 and 3 day animals, suggesting the loss of a higher sensitivity state of the tissue. Pirenzepine and mecamylamine cause steepening in 3 day treated animals, suggesting the possible involvement also of ganglionically induced release of acetylcholine as part of this high sensitivity process. K-induced contractions are in large part the result of evoked acetylcholine release from nerve terminals, since a large portion of the contraction is blocked by atropine. In 3 day DFP-treated animals the dose-response curve for K-induced contractions is shifted to the right, although by 7 days recovery from the decreased K-sensitivity has occurred. These shifts occur whether atropine is present or not. No shifts in the histamine dose-response curve for contraction were observed. The membrane potential of tracheal smooth muscle cells if -59 mV in control animals. After 7 days of DFP treatment the muscle is hyperpolarized to about -66 mV.

In addition to receptor down-regulation, other changes in tissue sensitivity occur after chronic organophosphate poisoning including a change in responses to in vitro DFP, to $K^{\oplus}$, and to the actions of inhibitors, as well as the cell membrane potential. The results illustrate the complexity of the alterations which occur during tolerance development.

AD_____

Tolerance Following Organophosphate Poisoning of Tracheal Muscle

Annual Report

Jerry M. Farley
Terry M. Dwyer
November 8, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Fredrick, Maryland 21701-5012

Contract No. DAMD17-83-C-3248

Dept. of Pharmacology and Toxicology
Univ. of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216-4505

Summary

Chronic reduction in acetylcholinesterase activity leads to an increase in the lifetime of acetylcholine, allowing its accumulation at synapses. The accumulation of the neurotransmitter leads to various signs and symptoms in vivo. The continual presence of acetylcholine also leads to changes in muscarinic receptor sensitivity of tissues. In part this is due to a down-regulation (decreased number) of muscarinic receptors. The following report deals with results from experiments designed to determine whether muscle muscarinic receptors and the tissue response to activation of these receptors change with chronic exposure to diisopropylfluorophosphonate (DFP). Measurements were made of the binding characteristics in control and DFP-treated animals. In addition, the isometric tension developed in response to various agents (acetylcholine, DFP, bethanechol, K^+) was determined in similarly treated animals. Finally, the membrane potential of muscle cells was determined in control and chronically treated animals, using microelectrode recording techniques.

Displacement of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding by agonists and antagonists suggests that the tracheal muscle receptors are of the M_2 subtype. Agonists apparently induce a state change in the receptor to a lower affinity form. In animals treated chronically with DFP, there is a decrease in receptor number which reaches 60-70% after 7 days. A 95% decrease in acetylcholinesterase activity also has occurred at this point.

The dose-response relationships for contraction as induced by acetylcholine show little shift in animals treated for up to 14 days with DFP. In vitro treatment with DFP in control tissue causes a spontaneous development of tension which can be blocked by atropine and pirenzepine and marginally by mecamylamine and is unaffected by tetrodotoxin (TTX).

In vitro DFP treatment does, however, shift the dose-response curve for acetylcholine to the left in both control and DFP-treated animals. This suggests that physiologically important amounts of cholinesterase still exist in the tissue even after 14 days of DFP treatment. In addition, the dose-response curves after in vitro DFP are much steeper in 7 and 14 day DFP-treated animals than in control, 1 and 3 day animals, suggesting the loss of a higher sensitivity state of the tissue. Pirenzepine and mecamylamine cause a similar steepening in a 3 day-treated animal, suggesting the possible involvement of ganglionically induced release of acetylcholine as part of this high sensitivity process. K-induced contractions are in large part the result of acetylcholine release from nerve terminals, since a large portion of the contraction is blocked by atropine. In DFP-treated animals (up to 3 days), the dose-response curve for K-induced contractions is shifted to the right, although by 7 days some recovery from the decreased K-sensitivity has occurred. These shifts occur whether atropine is present or not. No shifts in the histamine dose-response curve for contraction were observed. Finally, the membrane potential of tracheal smooth muscle cells is -59 mV in control animals and unaffected by DFP treatment until 7 days of treatment are completed, at which time the muscle is hyperpolarized to about -66 mV.

Thus it appears that in addition to down-regulation, other changes in tissue sensitivity occur after chronic organophosphate poisoning. These changes include a change in response to in vitro DFP, to K^+, and to the actions of inhibitors and in the cell membrane potential. The results point to the complexity of the system even when isolated tissues are used.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Table of Contents

List of Figures and Table

INTRODUCTION

Down-regulation of Muscarinic Receptors

When a tissue containing muscarinic receptors is exposed for an extended period of time to muscarinic agonists, generally the responsiveness of the tissue to the agonist decreases. A primary factor in the decreased tissue responsiveness is down-regulation of muscarinic receptors. This has been demonstrated in brain (1), vas deferens (2;3), AtT-20 pituitary tumor cells (4), trachea and other tissues. A decrease in receptor number with little change in affinity occurs in response to the various treatments used in these investigations. The down-regulation of receptors is related in some way to their activation, since muscarinic antagonists do not cause loss of receptors and can block the down-regulation caused by agonists (5).

In vitro exposure of a tissue to a muscarinic agonist induces a rapid (<30 min) and a more slowly developing loss of receptors from the cell surface. The rapid loss of receptors may result from a desensitization process related to an increase in the cyclic nucleotides cyclic GMP and cyclic AMP (6). Shifts in muscarinic receptor affinity (high to low) are also associated with the rapid down-regulation and may be related to dissociation of the Gi protein a guanine nucleotide (GTP) binding protein (Gi protein) associated with the recptor(7). The loss of receptors after long-term agonist exposure (e.g., 3 hours) from the cell is brought about by internalization of the receptors (2);(6) followed by degradation (8). Higuchi et al., (2), have shown that exogenously applied 8 BrcAMP, dibutyryl cAMP or dibutyryl cGMP did not increase the magnitude of the long-term down-regulation of muscarinic receptors in vas deferens; however, they did not measure the rate of this process. Agents such as cytochalasin B and vinblastin, which interfere with microfilament or microtubule function, inhibit the down-regulation of muscarinic receptors (2). It has been demonstrated that calcium is required for the recovery of receptors from down-regulated tissue (9). Thus the intracellular events which mediate or lead to the eventual down-regulation of muscarinic receptors are poorly understood.

While it is well documented that decreases in muscarinic receptor number occur after long-term agonist exposure, other changes which may also occur in the tissues are not well documented. Recently it was shown that down-regulation of muscarinic receptors in the AtT-20 pituitary cell line is associated with an increase in forskolin-stimulated cAMP formation (4). The increase in cAMP may be related to a loss of the G_i protein and/or increased synthesis of adenyl cyclase or both. We have demonstrated in pig trachea from animals treated daily with diisopropylfluorophosphonate (DFP) that a down-regulation in muscarinic receptor number occurs. There is about a 70% decrease in receptor number after 7 daily injections of DFP. In spite of this down- regulation, the contractile dose-response relationship to acetylcholine (ACh) or bethanechol shows little or no rightward shift. This suggests either 1) that extremely large numbers of spare receptors exist or 2) that other changes in tissue sensitivity occur during down-regulation. By contrast, the vas deferens shows a rightward shift in the ACh dose-response relationship with down-regulation (2). There is a hyperpolarization (by -6 mV) of the tracheal muscle cell membrane after 7 days, treatment of the animals with DFP, suggesting that some membrane properties change.

While little is known about other tissue changes which occur in response or in addition to down-regulation, much is known about tissue responses during up-regulation of receptors induced by denervation (10). For example, in vas deferens there is a leftward shift in the dose-response for contraction induced by methacholine (11), an 8 mV depolarization of the cells (Goto et al., 1978), an increase in length constant and a shift to a more negative potential of the threshold potential for action potential initiation (13). Some of these changes are related to the ionic properties of the membrane, although other changes (notably in cell-cell coupling) also occur. Thus it seems plausible that changes in the ionic properties of the membrane or second messenger-mediated events occur in response or in addition to the down-regulation of muscarinic receptors caused by muscarinic agonists.

METHODS

Contraction and Biochemical Studies

Treatment of Animals: Male weanling swine (Yorkshire, white), 5-15 kg, purchased from local suppliers, were used throughout this study. Swine were fed standard laboratory chow and given tap water, ad libitum, and were housed indoors in animal facilities with automatically controlled temperature and light cycle.

Swine were injected daily with DFP at the same time (9:00-9:30 AM). Sustained inhibition of cholinesterase was produced by intramuscular injections of DFP dissolved in normal saline(pH 5.5); 2 mg/kg was administered initially, followed by maintenance doses of 1 mg/kg DFP.

The pigs were sacrificed by severing the spinal cord near the base of the skull, using a captive bolt pistol. The tracheae were quickly isolated and transferred into oxygenated (95% O_2 and 5% CO_2) Krebs modified Ringer medium (14). The tracheae were maintained in this medium throughout the experiment. The tracheae were opened by cutting along the surface opposite to the region of attachment of the muscle to the tracheal cartilage. The tracheal epithelia were carefully peeled off with a forceps, and then the muscles were freed from all the adhering connective tissue. The cleaned tracheae were then cut into individual rings with a razor blade. Each ring was suspended in a separate muscle chamber for the recording of contractions.

The setup used for this study consisted of eight muscle chambers (acrylic tubing) installed in a series in a large rectangular tank. Water was circulated constantly in the tank at 37°C, using a Haake A81 thermostatic water bath (Hoake, Berlin, W. Germany). In this way the Ringer medium in the muscle chambers could be maintained at 37°C throughout the experiment. Inside the muscle chambers the tracheae were maintained in the Ringer medium, which was constantly bubbled with 95% O_2 + 5% CO_2.

Each tracheal ring was hooked in the muscle chamber to a Grass force transducer (Grass Inst., Co.), using a fine silk thread. The muscle contraction was recorded as tension in grams on both digital and analog scales, using a "Dianachart" recorder, datalogger (Dianachart Corp, Rockway, NJ).

DFP Treatment: DFP was administered to the pigs both in vivo and in vitro. In vivo administration was done by injecting the pigs with DFP for up to 14 days, at doses of 2 mg/kg on the 1st day and 1 mg/kg on the remaining days. In pigs that showed symptoms of sickness, the dose was reduced to 0.5 mg/kg from the 5th day onwards. In the present study, the pigs were injected with DFP and sacrificed at 2 hours, 1 day, 3 day, 7 day and 14 day intervals.

A baseline tension of 3 grams was applied to the muscle strips and they were given a stabilization time of 1 hour, with 2 to 3 changes of the Ringer medium during that period. The muscles were then used to generate dose-response curves or treated with 100 μM DFP, prepared in Krebs-Ringer medium, for 20 to 30 min. In vitro effects of DFP on the tracheal smooth muscle were studied by treating the muscle with the agent in the muscle chamber. The muscles were then washed with Krebs-Ringer 3 times and given a recovery time of 5 min before treating them with other agents.

ACh Effects: The effects of ACh were studied at concentrations from $1x10^{-11}$ M to $1x10^{-3}$ M. A stock solution of 1 M ACh was prepared in the Krebs-Ringer medium and diluted to the appropriate concentration with oxygenated warmed (37°C) Krebs-Ringer just before use. At each concentration of ACh, the muscle was allowed to reach its peak contraction and then washed with Ringer and allowed a recovery time of 5 min. Up to an ACh concentration of $1x10^{-5}$ M, this recovery time was enough to bring the muscle back to the baseline tension. However, after the treatment with $1x10^{-4}$ M ACh, repeated washes with Krebs-Ringer and greater recovery time (15 to 30 min) were required to bring the muscle back to the baseline tension.

Effects of Bethanechol, Histamine and K^+: The effects of bethanechol, histamine and K^+ were studied the same way as those of ACh. Stock solutions (1 mM) of bethanechol and histamine were prepared in Krebs-Ringer medium and aliquoted directly into 10 ml of medium in the muscle chamber to get the appropriate concentrations. The dose response to K^+, was generated by substituting the Na^+ in the Krebs-Ringer with an equivalent amount of K^+ to increase the K^+ concentration. This procedure maintained the osmotic pressure constant. In the present study bethanechol and histamine were used at concentrations from $1x10^{-9}$ to $1x10^{-2}$ M, while K^+ was used from 10 mM to 100 mM.

Effects of Mecamylamine, Pirenzepine and Atropine: Studies were performed of the actions of mecamylamine, pirenzepine and atropine on the spontaneous contractions in the tracheal smooth muscle in response to the treatment with DFP and ACh.

Also studied were the effects of atropine on the contractions in response to K^+. In the case of contractions to DFP and ACh, a final concentrations of mecamylamine ($1x10^{-4}$ M) or pirenzepine ($3x10^{-7}$ M) was added to the medium in the muscle chamber 5 min after the termination of DFP treatment. Thereafter these agents were continuously present in the chambers, throughout the remaining course of the experiment, i.e., during the ACh treatment as well as during the recovery in Krebs-Ringer.

On the other hand, atropine (2 μM) treatment was given only once (for 5 min) either before or after treating the muscle with DFP in vitro, and the agent was not present during the rest of the course of the experiment. In the

contractile studies with K^+, however, atropine (0.2 μM) was present in the muscle chamber throughout the experiment.

Dose-response curves for the effects of ACh, bethanechol, histamine and K^+ were plotted as percent of maximum contraction for each muscle.

Tissue Preparation: Tracheae, about 15 cm long from the larynx to the bifurcation, were removed from swine immediately after sacrifice and placed in 50 mM Na-K phosphate buffer (pH 7.4). The loose fat, connective tissue and epithelium were removed. The smooth muscle was then dissected free. The isolated smooth muscle was weighed, placed in 10 volumes of 50 mM Na-K phosphate buffer, and homogenized with a polytron (Kinematica GmbH, Switzerland; setting 10, 2 min). The homogenate was centrifuged at 500 x g for 10 min; the pellet was discarded and the supernatant was centrifuged at 35,000 x g for 20 min to obtain the membrane pellet. The resultant pellet was suspended in an appropriate volume of 50 mM Na-K phosphate buffer, which made the protein concentration of homogenate 0.02-0.3 mg/ml. This preparation was used for the binding assay without further processing. The tissue was stored frozen at -80°C until the binding assays were performed.

Cholinesterase Assay: The homogenates of tracheal smooth muscle were kept at 0°C and cholinesterase activities measured within 4 hours of sacrifice. Total cholinesterase was assayed by the colorimetric method of Ellman et al. (15), as modified by Benke et al. (16), using S-acetylthiocholine iodide as a substrate. The reaction involves the formation of the colored 5-thio-2-nitrobenzoate anion by the reaction of released thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The procedure for AChE assay is as follows: An aliquot of homogenate of tracheal smooth muscle or striatum (200 μl), 20 μl of 1.0 M S-acetylthiocholine iodide and 100 μl of 0.1 M DTNB were added to a cuvette containing 2.5 ml of 0.1 M phosphate buffer (pH 8.0). The absorbance was scanned for at least 10 min by a spectrophotometer (Gilford Instrument Co.) at 412 nm. The initial absorbance, as well as a reagent blank absorbance, was subtracted from the final reading. The activity of acetylcholinesterase was expressed as μmole S-acetylthiocholine iodide hydrolyzed/min/mg protein. The protein concentration was determined by the method of Lowry et al. (17), using bovine serum albumin as the standard. The butyrylcholinesterase assay was similar to that for AChE except that butyrylthiocholine iodide was used as a substrate. It should be noted that butyrlcholinesterase activity was much lower than the total cholinesterase activity that measured using acetylcholine.

Muscarinic Receptor Assay: The frozen tissue was thawed and homogenized with a polytron (Ultra-Turrax, West Germany; setting 60, 1 min). The [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding assay was performed according to the method of Yamamura and Snyder (18) with small modifications as described by Yang et al. (19). Briefly, in the standard assay, 0.2 ml of homogenate was added to a 10 ml test tube that contained 1.8 ml of 50 mM Na-K phosphate buffer (pH = 7.4) with varying concentrations of [^{3}H]QNB. The nonspecific binding assay was determined in the presence of 10 μM atropine sulfate. The assay was performed in triplicate. For equilibrium binding this mixture was incubated at 37°C for 45 min. The reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters. The filters had been previously soaked in 0.05% polyethyleneimine for 5 min and rinsed 4 times with 50 mM Na-K phosphate buffer to reduce nonspecific binding to the filter.

After the reaction mixture was filtered, the filters were rapidly washed 4 times with 5 ml of chilled 50 mM Na-K phosphate buffer. Each filter was placed in a vial containing 5 ml of scintillation solution and the radioactivity was determined by liquid scintillation counting (Tracor Analytic Mark III). The amount of specific binding was calculated as the total binding minus the binding in the presence of 10 μM atropine sulfate.

Down-regulation of Subtypes of Muscarinic Receptors: As noted earlier muscarinic receptors can be divided into at least two subtypes. In addition the subtypes generally have more than one substate with which agonists interact. Thus it is important to determine whether the subtype or substates for agonists of muscarinic receptors is altered by acute or subacute organophosprate treatment. Therefore to determine whether subtypes of muscarinic receptors are present in tracheal smooth muscle and whether one or whether both subtypes or substates of muscarinic receptors exist or are down-regulated in DFP-treated swine, a series of competition experiments were performed. The competing ligands chosen for this purpose were pirenzepine, selective M_1 antagonist; carbachol, selective M_2 agonist. Atropine, which is nonselective for both subtypes, was used to determine the change in the affinity of receptors.

An alternative method for receptor binding assay was used in these experiments. In the standard assay, 0.2 ml of homogenate was added to a 10 ml test tube that contained 1.8 ml of 50 mM Na-K phosphate buffer with a [^{3}H]QNB concentration of approximately 400 pM and varying concentrations of competing unlabeled inhibitor. All the conditions were the same as those described for the muscarinic receptor assay except that the assays were performed in duplicate. Following incubation, the reaction mixture was collected and washed by a cell harvester (Brandel Co., Gaithersburg, MD) as described by Bradford and Cidlowski (20). A piece of Whatman GF/B glass fiber filter, which was treated as described in the previous section, was placed in the cell harvester with 50 mM Na-K phosphate as the wash buffer. After filtration the filter was washed with the cell suction probe under vacuum. The total time for washing 24 samples did not exceed 1 min. The areas of the filter that contained reaction mixture were removed with a forceps. Each filter was placed in a scintillation vial containing 5 ml of scintillation solution and the radioactivity was determined by liquid scintillation counting (Tracor Analytic Mark III). The amount of specific binding was calculated as the total binding minus the binding in the presence of 10 μM atropine sulfate. The maximal number of binding sites (Bmax), equilibrium dissociation constant (K_D), and subtypes of muscarinic receptors were calculated by the equations described in the next section.

Analysis Binding Data: K_D and Bmax values were calculated using the method of Scatchard (21) by unweighted linear regression analysis of the transformed data. Subtype analysis was performed by fitting the competitive inhibition curves with either a one- or two-binding-site model, using an iterative least squares computer fit (program was provided by Dr. Marcy Petrini). The equation used was described by Hoyer et al. (22) as follows:

$$B = \sum_{i=1}^{n} \frac{Bmax_i}{1 + (K_D/L)(1 + I/K_{Ii})}$$

where K_D and L are the equilibrium dissociation constant and concentration of free ligand, $Bmax_i$ is the maximal number of binding sites in state i (i=1 or 2), and I and K_{I_i} are the free concentrations and the equilibrium dissociation constant, respectively, of the competing ligand for the state i. The model which most accurately describes the data was determined by Fischer's F-test (23).

Microelectrode Methods

A single tracheal ring was used in the measurement of resting membrane potential. The muscle strip was pinned down in an acrylic plastic chamber with the lumen side up. A small amount of tension was applied to the muscle. The connective tissue was carefully cleaned from the surface of the muscle with a pair of fine forceps. The modified Krebs-Ringer medium (14), which was used as the bathing medium, was maintained at 37°C.

The chamber which was used in this experiment consisted of a central muscle chamber and two small side chambers for inflow and outflow of the perfusion fluid. The total volume of the chamber was 9 ml. The bathing medium was aerated with 95% O_2-5%CO_2 before perfusion. The criterion for successful impalement of a cell was a stable membrane potential record for at least 1 min.

Glass microelectrodes were pulled on a horizontal two stage puller (Industrial Science Associates, Inc., Ridgewood, NY) from 1 mm microfilament glass capillary tubing. These were filled with the solution containing 1 M KCl and 2 M KAc, using a 3 inch needle syringe. Microelectrodes with resistances from 70 to 80 megohms were used to impale cells. A microelectrode manipulator (Custom Medical Research Equipment, Glendora, NJ) capable of <2 μm minimum movement was used to drive the electrodes into the cell. A high impedance input amplifier (Model M-707 Microprobe System, W-P Instruments, Inc., New Haven, CT) was used to measure the resting membrane potential. The output of this amplifier was connected to a Gould 2200S chart recorder (Gould Inc., Cleveland, OH) and 5111 storage oscilloscope (Tektronix, Inc., Beaverton, OR). The half-cell electrode used to connect the microelectrode and bath to the amplifier was sintered silver-silver chloride pellets.

Isolation of Tracheal Smooth Muscle Cells

The isolation procedure we used is similar to that of Bitar and Makhlouf (24), Benham and Bolton (25), and Mitra and Morad (26). The primary emphasis in the technique is on the isolation of relaxed elongate cells.

Tracheae were rapidly removed, using sterile technique, and placed in a 50 ml sterile tube filled with sterile physiological saline containing 1:1000 ticarcillin, 1:4000 tobramycin and 0.2% Fungizone. Tracheae were removed from the tube in a laminar flow hood and were handled using sterile technique. All solutions were cold-sterilized by filtration through 0.2 μ disposable filter units. Tracheae were cut open longitudinally through the cartilage rings and pinned out luminal side up. The epithelia were stripped away, using forceps, and the muscles were cleaned. The tracheae were then turned over and cleaned of connective tissue. This leaves a flat sheet of muscle connected to the cartilage rings. The muscles were cut free at their insertions and minced.

The minced muscle was transferred to the dissociation medium containing 0.2% collagenase (Type 1), 0.01% DNAase and 0.1% bovine serum albumin as well as the antibiotics and antimycotic agents in physiological saline. The physiological saline contained (mM): 120 NaCl, 2 KCl, 2 $CaCl_2$ or 0.2 $CaCl_2$, 10 glucose and 5 HEPES (pH 7.4). This procedure is similar to the method of[2] others. The tissue pieces were then gently agitated at 37°C in a rotary incubator in an atmosphere of 95% O_2-5% CO_2. All subsequent handling of the tissue and cells was performed so as to minimize the shear forces on the isolated cells. The cells isolated in this manner are elongate, relaxed and easily identified as smooth muscle cells. The cells were diluted to a density of approximately $1x10^6$ cells/ml. The isolation procedure may be carried one step further to isolate, purify and concentrate the smooth muscle cells, using a Percoll density gradient (Pharmacia). Percoll is a nontoxic colloidal suspension with low osmotic pressure that can be made up in physiological salt solution to provide solutions of well-defined density. Discontinuous or continuous density gradients can be formed. In order to reduce the stress of centrifugation on these fragile cells, a layer of high density Percoll ($1.1-1.2$ gm/cm^3) can be used as a buffer to allow concentration of cells without the mechanical stress of pelleting.

Isolated relaxed cells were used in experiments, since these should be physiologically the most normal. All procedures used were intended to maximize the percentage of relaxed elongate cells. In three preliminary experiments, although were able to we isolate many relaxed tracheal cells, only 30% of the cells are relaxed. This procedure will be improved during the next year.

RESULTS

Binding: Figure 1 shows the decrease in receptor number and decrease in acetylcholinesterase in the trachea which occurs when animals are exposed daily to DFP. The down-regulation was complete by day 7, with the receptor number being decreased by 60-70%. Acetylcholinesterase was reduced in a similar fashion, the maximal inhibition being about 95%. No change in the K_D for QNB occurred even though receptor number had been greatly reduced (51 ± 4 PM; control;49 ± 8 PM, 7 day). After cessation of DFP treatment for 3 days 3* in Figure 1), little recovery in receptor number occurred. However, after 14 days of recovery (14* in Fibure 1) the receptor number had returned to approximately 65% of control.

Pharmacologically the muscarinic receptor appears to be of the M_2 subtype. Displacement inhibition curves of QNB binding are shown in Figure 2. QNB, a nonselective antagonist; pirenzepine and quinuclidinyl xanthene-9-carboxylate (QNX), selective M_1 antagonists; carbachol, a selective M_2 agonist; and pilocarpine, an M_1 agonist, were used as displacing agents. The displacement of [^{3}H]QNB for the antagonists was best fit assuming competition for a single class of binding sites of the M_2 subtype. The data for both agonists were best fit assuming a two-binding-site model. The compilation of these data is given in Table 1. Thus it may be that agonists induce a state change in the receptor molecules.

Contraction

Muscarinic Agonists: Figure 3A is an illustration of an ACh-induced isometric contraction in a tracheal muscle strip. The contraction developed slowly requiring 1 to 5 minutes to reach a steady state contraction. The contraction could be sustained at a steady level for 30 minutes as long as agonist was present in the bath. was sustained. The dose-response relationships for ACh-induced contractions are shown in Figure 3B for control animals and animals treated with DFP. It is surprising that there was a leftward shift (as compared with control) in the dose-response curve even after 14 days of DFP treatment. This occurred despite the 60-70% decrease in receptor number and the great decrease in acetylcholinesterase activity. In fact, 1, 3, 7 and 14 day treated muscle gave almost identical dose response relationships.

In vitro DFP caused slowly developing (10-20 min time to reach a maximum osustained contractions of tracheal muscle from control animals. This contraction was blocked by atropine but not by tetrodotoxin (TTX) (1×10^{-6} M). Pirenzepine, a selective M_1 antagonist (3×10^{-7} M), also blocked the response. Finally mecamylamine, a selective ganglionic, nicotinic receptor blocker, inhibited the DFP-evoked contraction part of the time. Thus the spontaneous contraction was caused by activation of muscarinic receptors, probably via ganglionic release of ACh.

In vitro DFP treatment (100 μM for 20 to 30 min) affected the dose-response curves as shown in Figure 4, causing them all to shift to the left. This demonstrates that even after 14 days of DFP treatment, there were substantial physiologically active amounts of acetylcholinesterase present in the intact muscle. Under these conditions there was a rightward shift in the dose-response relationship at 7 and 14 days as compared with control DFP-treated muscle. The shift to the right at 7 and 14 days was not parallel, however; rather it appeared as a steepening of the dose-response relationship. After 1 and 3 days of DFP, no shift was evident. Thus it seems that an apparent high affinity state or high sensitivity state of the muscle was lost with chronic DFP treatment.

Pirenzepine and mecamylamine were added to the bath and dose-response relationships determined for ACh. Mecamylamine (10^{-4} M) had no effect on the dose-response in 3 day DFP-injected animals (Figure 5). However, if this tissue was treated with DFP in vitro, a rightward shift and steepening (loss of low concentration effects of ACh) were evident. Pirenzepine (a selective M_1 antagonist) at 3×10^{-7} M had a similar but more dramatic action. In control and 3 day DFP-treated tissue, there was a parallel shift in the dose-response caused by pirenzepine (Figure, 6A,B), which suggests a purely competitive inhibition. While pirenzepine is called a selective M_1 antagonist, it does block M_2 receptors, albeit with a lower affinity than M_1. However, in tissue treated in vitro with DFP, there was no longer a parallel shift to the right caused by pirenzepine but rather a steepening of the dose-response curve. This was especially evident in the control animals' tissue (Figure, 6A) and is suggestive of possible ganglionic involvement in the contractile response.

If pigs treated with DFP for 14 days were allowed to recover for 14 days, the response of tracheal muscle to ACh and in vitro DFP was exactly that of control animals (Figure, 7).

Potassium: Increasing extracellular potassium caused tracheal muscle to contract (Figure, 8A). The contraction developed slowly and was sustained. Atropine (0.2 μM) was used to block the effects of potassium-induced release of ACh from nerve terminals. This converted the tonic contraction into a phasic contraction (Figure, 8B). Thus it appears that potassium-induced contractions were due in large part to the release of endogenous ACh.

The dose-response curves for peak potassium-induced contraction were measured in animals treated daily with DFP. The results are shown in Figure, 9A. There was a shift to the right in the dose-response relationship up to at least 3 days of DFP treatment. This suggests a decrease in the sensitivity of the muscle to potassium. By the 7th day of DFP treatment, however, there was a shift back toward the control dose-response. In vitro application of atropine (0.2 μM) did not change the shifts of the dose-response curves (Figure 9B), suggesting that the shift observed is due to changes in the sensitivity of the muscle to potassium or some other change intrinsic to the muscle tissue and not to the ganglionic release of potassium.

Histamine: Histamine contractions also were induced; however, the contractions were very weak. Figure, 10 shows the dose-response for histamine-induced contractions in control animals and DFP-treated animals. There was no effect of DFP treatment on the histamine-evoked response.

Membrane Potential Measurements

Membrane potential was measured using glass microelectrodes (60-100 megohms). In normal Krebs Ringer the membrane potential was about -59 mV (Figure, 11). This potential was not affected by up to 3 day treatment of animals with DFP. However, by 7 days there was a hyperpolarization of the muscle cell by about 7 mV, bringing it to -66 mV. Thus it appears that basic changes in membrane properties occurred.

DISCUSSION

The effect, of DFP on the binding properties of muscarinic receptors, contractile response of the muscle and membrane potential have been presented. Several of the findings stand out. First, even in the face of the large decrease in muscarinic receptor numbers (i.e., Δ70%) caused by daily DFP treatment, the dose-response relationships for ACh and bethanechol are relatively unaffected. This is quite different from dose-response relationships obtained in vas deferens after muscarinic receptor down-regulation (3,9).

The reasons for the lack of shift on the dose-response could be quite complex. For example, histologically the tracheal muscle is known to be innervated by very short postganglionic parasympathetic fibers, the ganglia being intimately associated with the muscle (27). Thus changes which occur in tracheal muscle responsiveness may in part be due to effects of DFP on the ganglia as well as the muscle. The possible involvement of ganglia is suggested by the ability of receptor blockers such as atropine (M_1 and M_2), pirenzepine (M_1) and mecamylamine (ganglionic nicotinic) to modify tracheal muscle contraction, especially after in vitro treatment with DFP. The ability of these agents to reverse the spontaneous contraction induced by DFP in control muscle is indicative of the probable involvement of ganglia in the tissue response. Finally, the fact that the contractile response to potassium is in large part due to the evoked release of ACh affirms to the importance of ganglia in tissue responsiveness.

Thus, since the ganglia or at least evoked release of ACh is involved in tissue responsiveness, any changes in ganglionic responsiveness would result in alterations in the muscle's response. Ganglia will be affected by DFP, since both nicotinic (28) and muscarinic (M_1) receptors (29) exist there.

The responsiveness of the muscle to potassium changes and the fact that membrane potential may be altered suggest that down-regulation is also coupled with changes in the properties of the muscle. All of these changes are probably related to the lack of shift in the dose-response relationship for contraction.

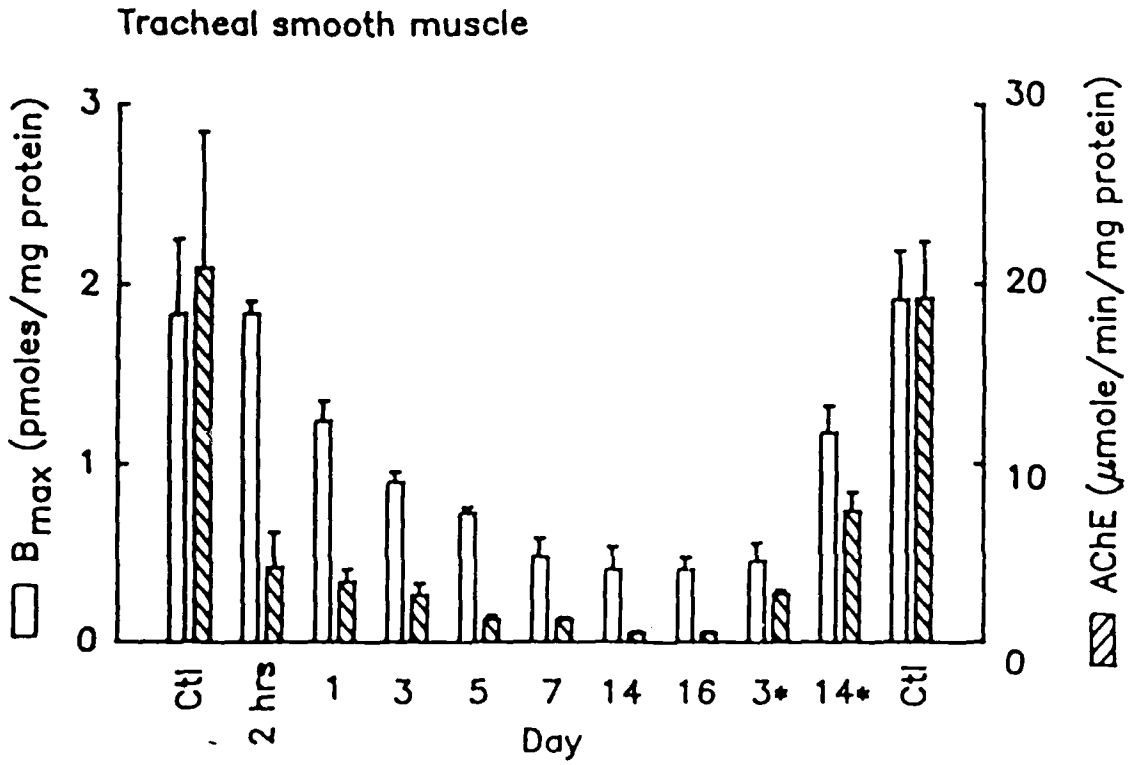


FIGURE 1

THE INHIBITION OF ACETYLCHOLINESTERASE AND DECREASE IN MUSCARINIC RECEPTOR NUMBER IN TRACHEAL SMOOTH MUSCLE AFTER CHRONIC DFP TREATMENT

The histograms above show the effect of chronic DFP treatment on the muscarinic receptor density (cross-hatched bars) and acetylcholinesterase (AChE) activity (open bars). There is a 60-70% decrease in receptor number and an approximate 95% decrease in acetylcholinesterase activity after 7 days of DFP treatment. The receptor binding and cholinesterase activity were determined 24 hours after the last injection to the animals.

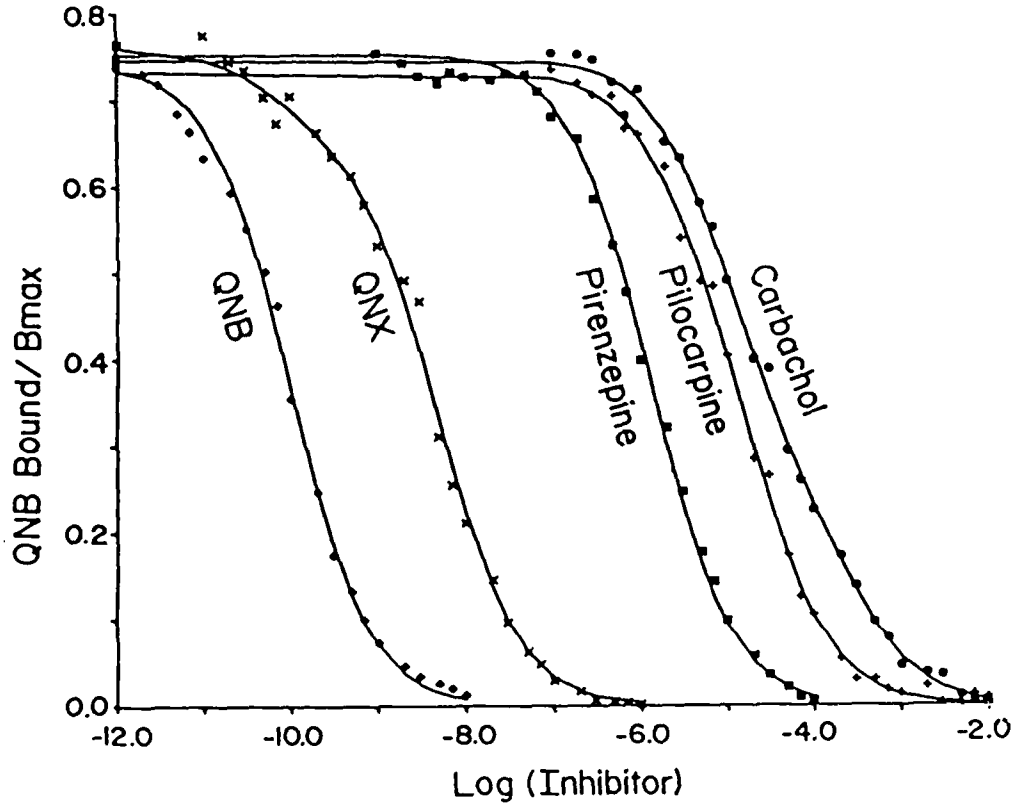


FIGURE 2

DISPLACEMENT ANALYSIS OF [^{3}H]QNB BINDING
BY VARIOUS MUSCARINIC LIGANDS

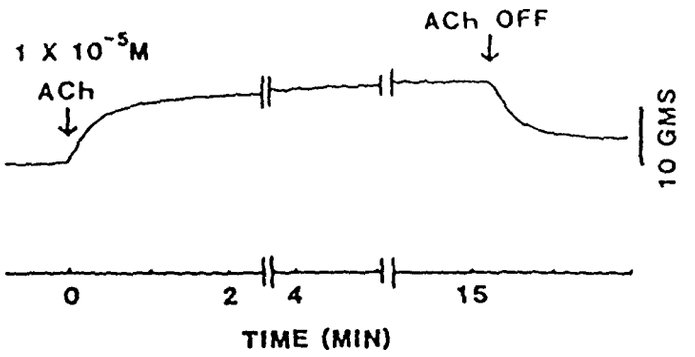
The displacement of [^{3}H]QNB by muscarinic ligands was performed using QNB, pirenzepine, and atropine (antagonists) and pilocarpine and carbachol (agonists). The solid lines are computer-generated best fits to a one-binding site model for antagonists or a two-binding site model for agonists. The receptor has characteristics consistent with the M_2 subtype. The calculated K_D and Bmax are given in Table 1.

TABLE 1. Binding parameters obtained by competitive inhibition experiments and analyzed by the one- or two-binding-site models[a]

Drug	K_{I1}[b]	K_{I2}[b]	$Bmax_1$[c]	$Bmax_2$[c]
ANTAGONISTS				
Pirenzepine	$(2.56\pm0.12)\times10^{-7}$	--	1.69±0.12	--
QNB	$(2.21\pm0.09)\times10^{-11}$	--	1.71±0.23	--
QNX	$(7.78\pm1.38)\times10^{-10}$	--	1.68±0.07	--
AGONISTS				
Carbachol	$(2.17\pm0.06)\times10^{-6}$	$(4.86\pm0.41)\times10^{-5}$	1.11±0.05	0.58±0.01
Pilocarpine	$(1.19\pm0.70)\times10^{-6}$	$(5.36\pm1.66)\times10^{-6}$	0.67±0.33	1.09±0.30

a. The binding parameters ± standard deviation were calculated by the nonlinear regression procedure for one-and two-site models.
b. K_I values are expressed as molar
c. Bmax is expressed as pmole/mg protein.

A.



B.

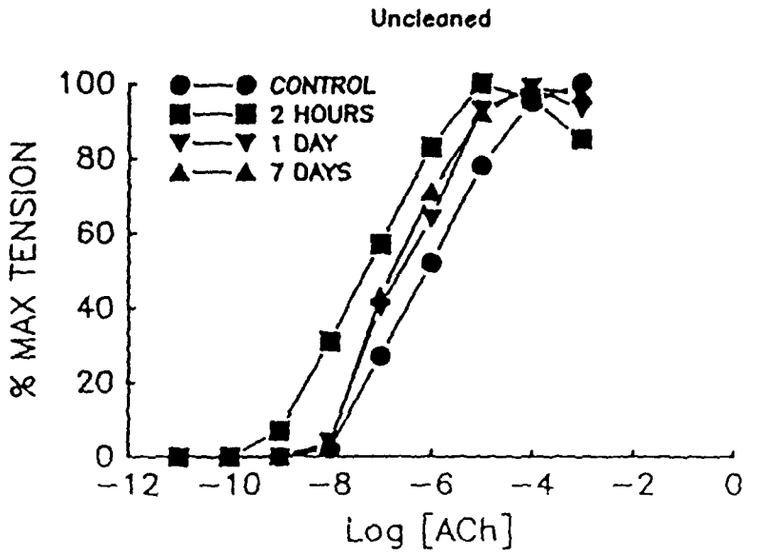


FIGURE 3

DOSE-RESPONSE CURVES FOR ACETYLCHOLINE-INDUCED CONTRACTIONS IN DFP-TREATED ANIMALS

A. The time course of an ACh-induced contraction

B. The dose-response curves for ACh-induced contractions (normalized to the maximal tension) are shown. These average curves were obtained from at least 5 animals and 10 muscles. The curves are labeled with the treatment group of animals from which the data were obtained (i.e., length of DFP treatment).

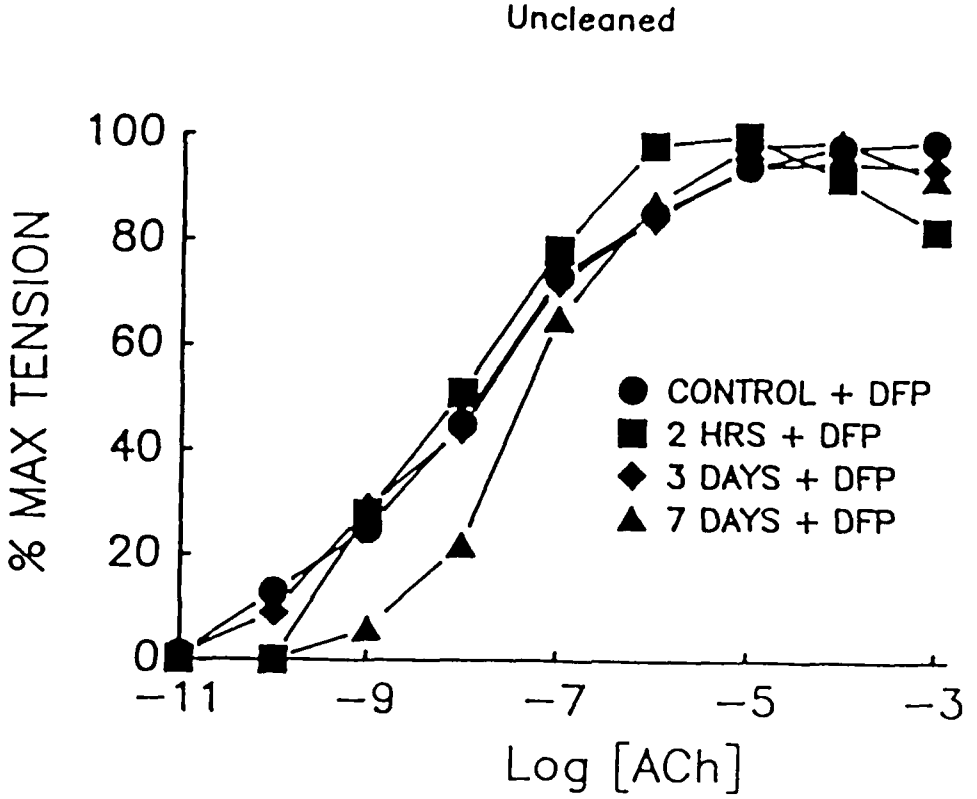


FIGURE 4

THE EFFECT OF IN VITRO DFP (100 μM) ON THE DOSE-RESPONSE CURVES FOR ACETYLCHOLINE

Dose-response curves for AChinduced contractions (normalized to maximal tension) from animals treated with DFP for the length of time shown and then pretreated again with DFP in vitro. At least 4 animals and 8 muscles were used to generate each curve.

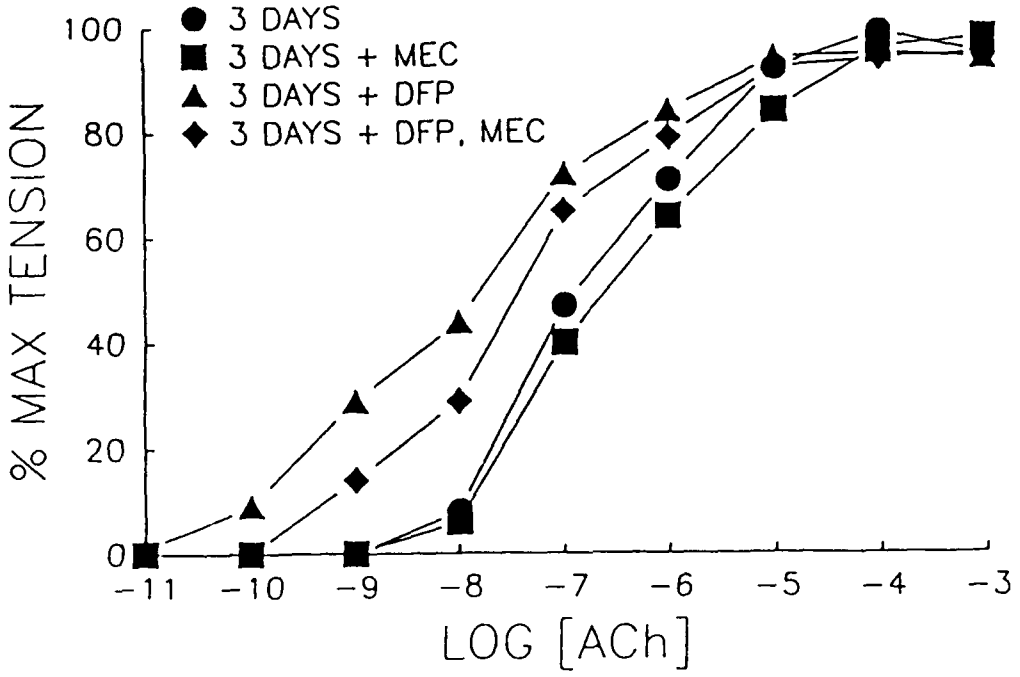
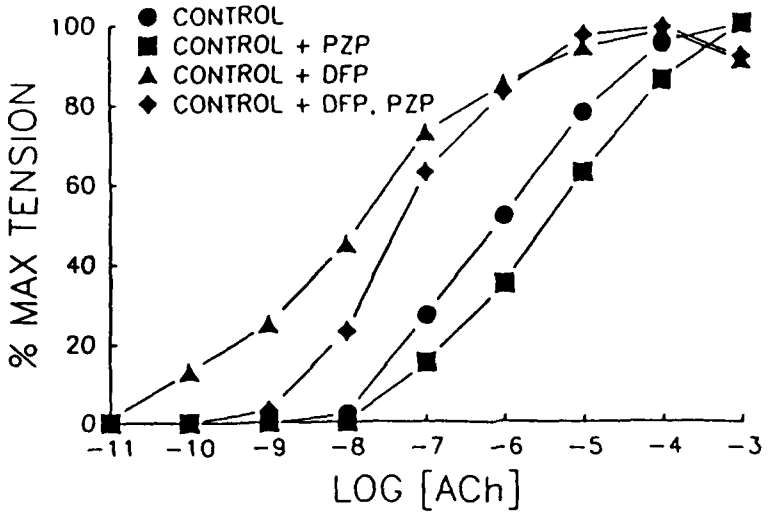


FIGURE 5

THE EFFECT OF MECAMYLAMINE ON THE DOSE-RESPONSE CURVES FOR ACETYLCHOLINE-INDUCED CONTRACTIONS

Dose-response curves for ACh-induced contractions (normalized to maximum tension) are shown above. Also shown is the effect of mecamylamine (MEC) (10 μM) on the dose-response relationships from animals treated with DFP for 3 days. The curves labeled with DFP were also treated with 100 μM DFP in vitro. Note the steepening of the curve in the presence of MEC in in vitro DFP-treated muscles.

A.



B.

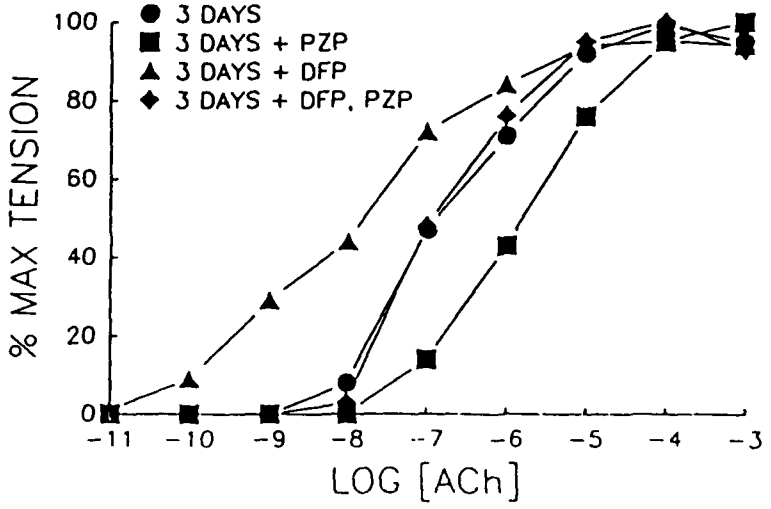


FIGURE 6

THE EFFECT OF PIRENZEPINE ON DOSE-RESPONSE RELATIONSHIPS FOR ACETYLCHOLINE

The dose-response relationships for ACh-induced contractions (normalized maximum tension) are shown above. The effects of pirenzepine (PZP)in control tissue and tissue treated with 100 μM DFP in vitro are shown in A. In B, the effects of PZP on tracheal muscle contraction from pigs treated 3 days with DFP are shown. Note the steepening of the curve in the presence of PZP in both A and B.

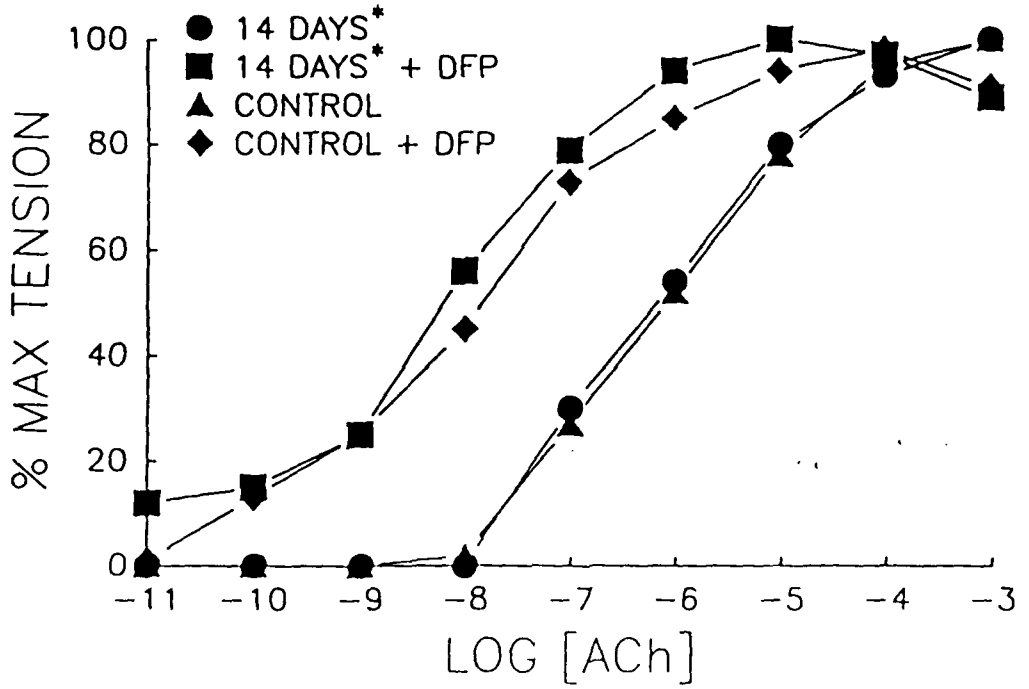


FIGURE 7

THE DOSE-RESPONSE RELATIONSHIP FOR ACETYLCHOLINE-INDUCED CONTRACTION: RECOVERY FROM DFP TREATMENT

Dose-response are shown for ACh-induced contractions from control animals or animals treated for 14 days with DFP, then allowed to recover for 14 days. Dose-response curves from untreated tissue and tissue treated with 100 μM DFP in vitro are shown.

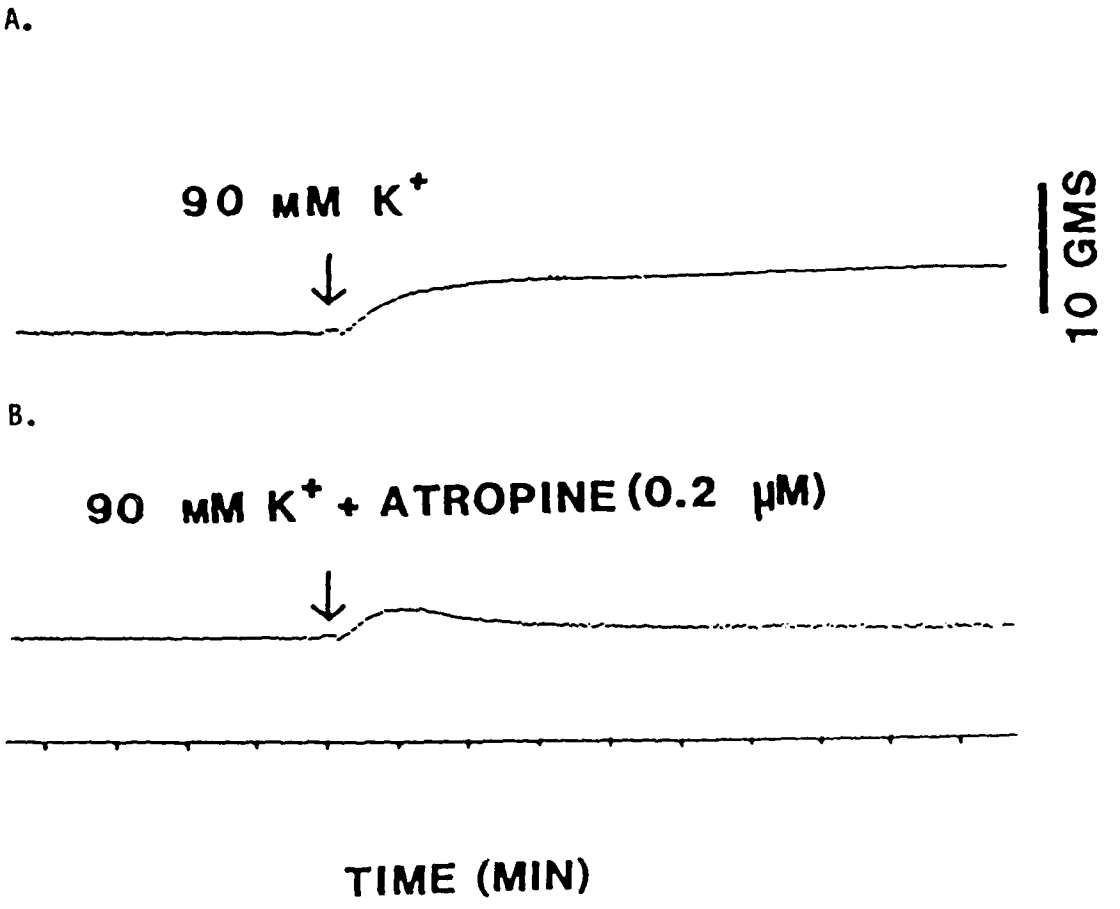


FIGURE 8

RECORDS OF K-INDUCED CONTRACTIONS

K-induced contractions are shown in control tissue (A) and in tissues pretreated with 1 μM atropine (B). Note the change in shape of the contraction in the presence of atropine.

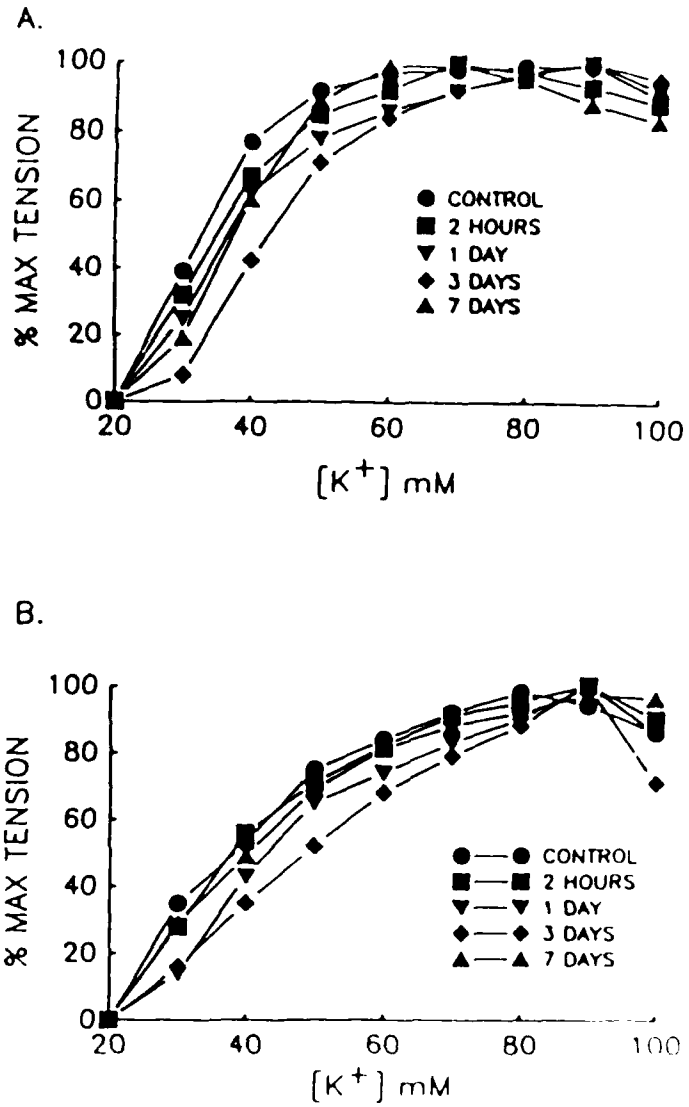


FIGURE 9

DOSE-RESPONSE RELATIONSHIPS FOR K^+-INDUCED TRACHEAL CONTRACTION

Extracellular K^+ was increased by replacing Na^+ with K^+ in the concentration given on the x-axes. The tension generated by each muscle was normalized to the maximal tension produced by that muscle. The average dose-response curves shown were obtained from 6-8 muscles from at least 3 pigs. The curves are labeled with the length of treatment of the animals with DFP.

A. Dose-response curves obtained in normal Krebs solution.
B. Dose-response curves obtained in the presence of 1 μM atropine.

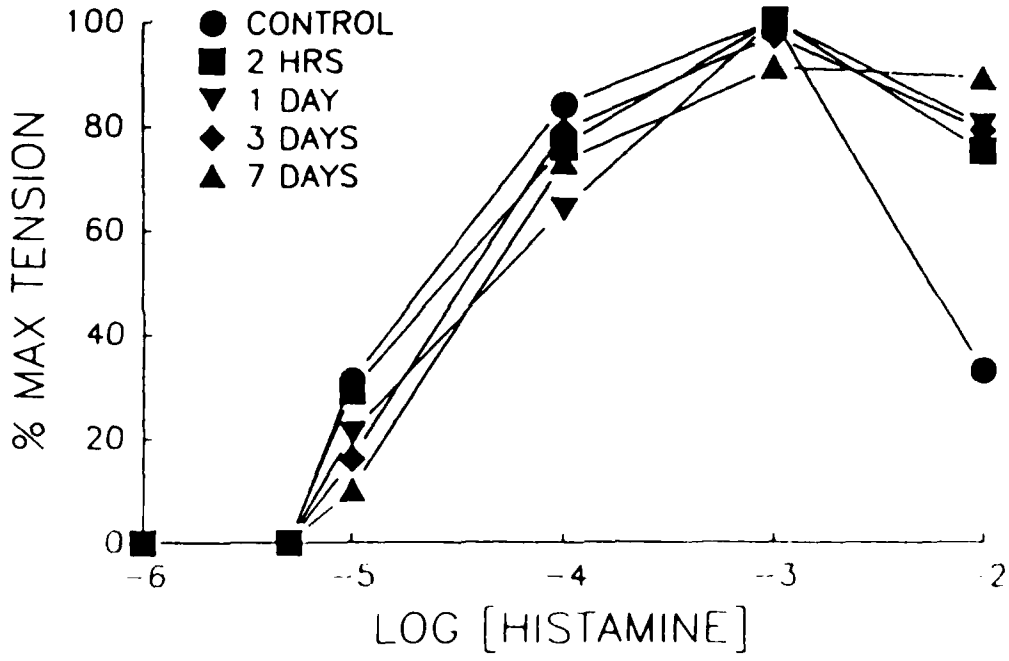


FIGURE 10

HISTAMINE DOSE-RESPONSE CURVES

Dose-response curves for histamine-induced contractions are shown. The dose-response curves were obtained in tissues from animals treated with DFP for the lengths of time shown. The contractions were normalized to maximum tension. No effect of DFP treatment on histamine action was observed.

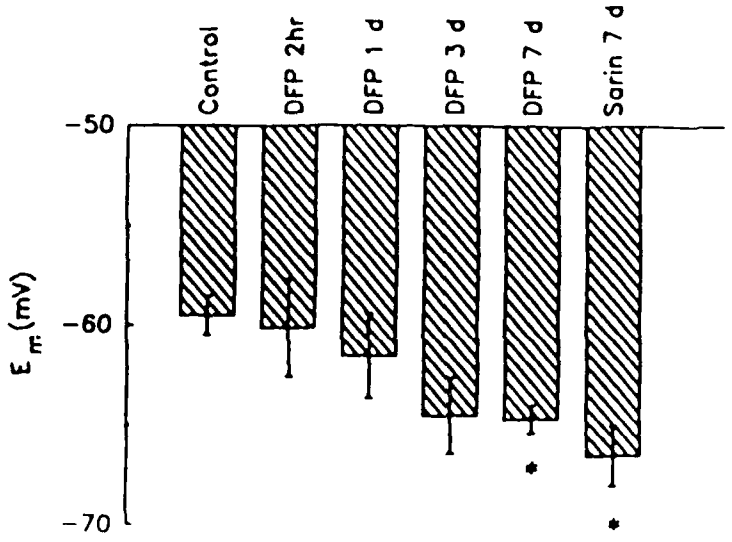


FIGURE 11

DFP TREATMENT AND MEMBRANE POTENTIAL

Tracheal muscle cell membrane potential was measured using glass microelectrodes. The bar chart above shows the mean ± standard error of the mean for cell potentials maintained stable for at least 1 min. There were at least 3 animals in each group. The length of DFP treatment is given above each bar graph. The * indicates a significant difference ($P < 0.05$).

REFERENCES

1. Lerer, B., H. Attman and M. Stanley (1984) Brief communication. Enhancement of memory by a cholinesterase inhibitor associated with muscarinic receptor down-regulation. Pharmacology Biochemistry and Behavior 21: 467-469.

2. Higuchi, H., K. Takeyasu, S. Uchida and H. Yoshida (1981) Receptor-activated and energy-dependent decrease of muscarinic cholinergic receptor in guinea-pig vas deferens. Eur. J. Pharm. 75: 305-311.

3. Higuchi, H., K. Matsumoto, S. Uchida and H. Yoshida (1983) Binding properties of muscarinic cholinergic receptors in long-term desensitization of smooth muscle. Jap. J. Pharm. 33: 702-705.

4. Heisler, S., D. Desjardins and M-H. Nguyen (1985) Muscarinic cholinergic receptors in mouse pituitary tumor cells: Prolonged agonist pretreatment decreases receptor content and increases forskolin- and hormone-stimulated cyclic AMP synthesis and adrenocorticotropin secretion. J. Pharmacol. Exp. Ther. 232: 232-238.

5. Siman, R.G. and W.L. Klein (1979) Cholinergic activity regulates muscarinic receptors in central nervous system cultures. Proc. Natl. Acad. Sci. 76: 4141-4145.

6. Feigenbaum, P. and E.E. El-Fakahany (1985) Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist. Possible involvement in desensitization of receptor function. J. Pharm. Exp. Ther. 233: 134-140.

7. Galper, J.B., L.C. Dziekan, D.S. O'Hara and T. W. Smith (1982) The biphasic response of muscarinic cholinergic receptors in cultured heart cells of agonists. JBC 257: 10344-10356.

8. Higuchi, H., K. Takeyasu, S. Uchida and H. Yoshida (1982) Mechanism of agonist-induced degradation of muscarinic cholinergic receptor in cultured vas deferens of guinea-pig. Eur. J. Pharm. 79: 67-77.

9. Higuchi, H., S. Uchida and H. Yoshida (1985) Recovery of the muscarinic cholinergic receptor from its down-regulation in cultured smooth muscle. Eur. J. of Pharmacol. 109: 161-171.

10. Fleming, W.W. (1976) Variable sensitivity of excitable cells: Possible Mechanisms and Biological Significance. Rev. Neurosci. 2: 43-90.

11. Goto, K., T. Masaki, A. Siato and Y. Kasuya (1979) Denervation-like supersensitivity in the rat vas deferens induced by local application of colchicine to the hypogastric plexus. JPET 209: 376-381.

12. Goto, K., D.P. West fall and W.W. Fleming (1978) Denevation-included changes in electrophysiologic parameters of the smooth muscle of the guinea-pig and rat vas deferens. JPET 204: 325-333.

13. Goto, K (1980) Electrophysiological analysis of colchicine-induced supersensitivity in the rat vas deferens. J. Physiol. 308: 465-477.

14. Farley, J.M. and P.R. Miles (1977) Role of depolarization in acetylcholine induced contractions of dog trachealis muscle. J. Pharmacol. Exp. Ther. 201: 199-205.

15. Ellman, G.L. K.D. Courtney, V. Andres and R.M. Feathersone (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95.

16. Benke, G.M., K.L. Cheever, F.E. Mirer and S.D. Murphy (1974) Comparative toxicity anticholinesterase action and metabolism of methylparathion and parathion in sunfish and mice. Toxicol. Appl. Pharmacol. 28: 97-109.

17. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

18. Yamamura, H.I. and S.H. Snyder (1974) Muscarinic receptor binding in longitudinal muscle of guinea pig ileum. Mol. Pharmacol. 10: 861-867.

19. Yang, C.M., J.M. Farley and T.M. Dwyer (1986) Biochemical characteristics of muscarinic cholinergic receptors in swine tracheal smooth muscle. J. Autonom. Pharmacol

20. Bradford, O.F. and J.A. Cidlowski (1984) A procedure for the measurement of hormone receptors using a cell harvesting machine. J. Receptor Res. 4: 727-739.

21. Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51: 660-672.

22. Hoyer, D., E.E., Reynolds and P.B. Molinoff (1984) Agonist-induced changes in the properties of Beta-adrenergic receptors on intact S49 lymphoma cells: Time-dependent changes in the affinity of the receptors for agonists. Mol. Pharmacol. 25: 209-218.

23. Zar, J.H. (1974) Biostatistical Analysis: Multiple regression and correlation, Prentice-Hall Inc., Englewood Cliffs, N.J. pp.252-280.

24. Bitar, K.N. and M. Makhlouf (1982) Receptors on smooth muscle cells: Characterization by contraction and specific antagonistics. Am. J. Physiol. 242: 400-407.

25. Benham, C.D. and T.B. Bolton (1983) Patch-clamp studies of slow potential-sensitive potassium channels in longitudinal smooth muscle cells of rabbit jejunum. J. Physiol. Lond. 340: 469-486.

26. Mitra, Rand M. Morad (1985) A uniform enzymatic method for the dissociation of myocytes from hearts and stomachs of vertebrates. AM. J. Physiol 249 (Heart Circ. Physiol. 18) H1056-H1010.

27. Baluk, P., T. Fujiwara and S. Matsuda (1985) The fine structure of the ganglia of the guinea-pig trachea. Cell Tissue Res. 239: 51-60.

28. Dotty, J.O. and Barnard, E.A. (1984) Nicotinic acetylcholine receptors: an overview. Biochem. Pharmacol. 33: 841-858.

29. Wilffert, B., D. Davidesko, A. De Jonge, M.J. Thoolen, P.B. Timmermans and P.A. Van Zwieten (1983) Role of ganglionic M-1 and M-2 receptors in the neuronal control of cardiovascular system of the normotensive rat as determined with pilocarpine. Eur. J. Pharmacol. 95: 109-116.

DISTRIBUTION LIST

4 copies: Commander
US Army Medical Research & Development Command
ATTN: SGRD-RMS
Fort Detrick
Fredrick, MD 21701

5 copies: Commander
US Army Medical Research & Development Command
ATTN: SGRD-PLE
Ft. Detrick
Fredrick, MD 21701

12 copies: Administrator
Defense Technical Information Center
Cameron Station
Alexandria, VA 22314

1 copy: Commandant
Academy of Health Sciences, US Army
ATTN: ASH-CMD
Fort Sam Houston, TX 78234

1 copy: Dean, School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20014

END

9-87

DTIC